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# Circadian Expression Patterns of Proteins in the Suprachiasmatic Nucleus

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# HONORS THESIS

**Jenna Elizabeth McKinnie**

## **Circadian Expression Patterns of Proteins in the Suprachiasmatic Nucleus**

### **Abstract**

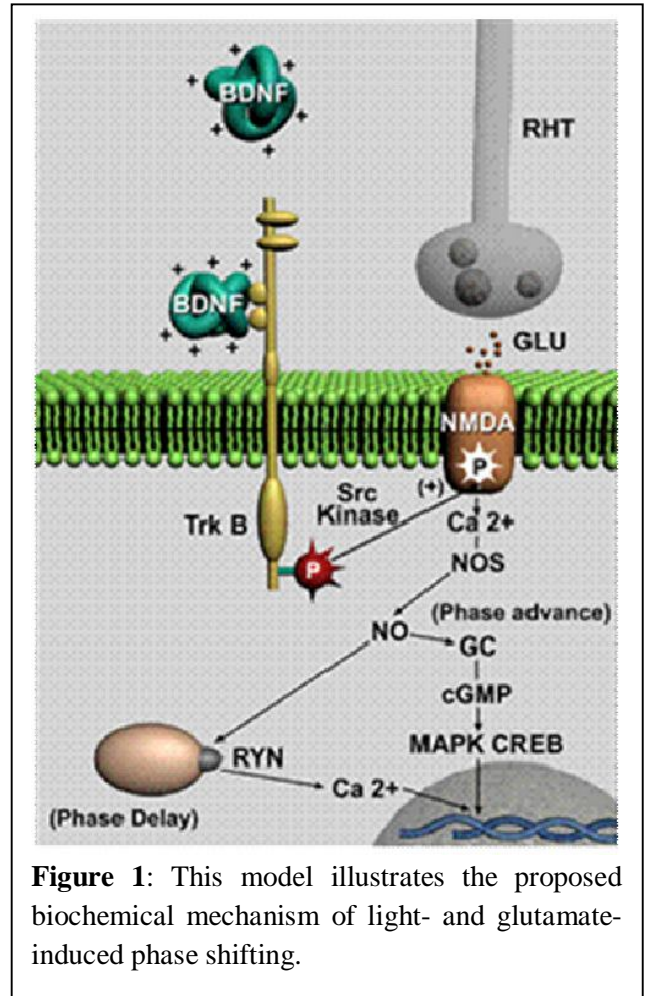
Previous studies indicate that several extracellular proteases are essential in the function of the central nervous system. Plasmin is formed when plasminogen is cleaved by tissue plasminogen activator (tPA). In the brain, plasmin catalyzes the formation of mature brain derived neurotrophic factor (mBDNF). Although it is known that tPA protein is abundant in the hypothalamus, little attention has been given to its function there. Based upon studies in the hippocampus, we hypothesized that tPA and its associated proteins modulate photic phase resetting of the mammalian circadian clock in the suprachiasmatic nucleus (SCN) by activating BDNF. We utilized an immortalized SCN2.2 cell line to examine tPA action within the SCN. This is an ideal model because we hypothesize that tPA and its associated proteins function in the extracellular space, and cell culture allows us to visualize and quantify proteins in the individual culture compartments—media, cells, and extracellular matrix (ECM). Thus, we can examine protein interactions and functions both intracellularly and extracellularly. We used Western Blot analysis of protein samples extracted from SCN2.2 cells to investigate baseline expression patterns of these proteins. Cultures were synchronized to the same phase by forskolin administration and then harvested and separated into fractions at three-hour intervals over a twenty-four hour cycle. We analyzed cell fraction samples for the presence of tPA and plasminogen activator inhibitor 1 (PAI-1) protein. Current data indicate that tPA is expressed rhythmically in the cell fraction, and that PAI-1 is not. Since these proteins likely function extracellularly, their relative levels of expression in the media and ECM fractions will provide important information regarding the functional interactions between these two proteins.

## Introduction

Within the mammalian hypothalamus, the suprachiasmatic nucleus (SCN) contains a circadian clock that regulates the daily timing of many physiological and behavioral rhythms (Klein et al., 1991). These rhythms are endogenously generated, and can be synchronized to environmental signals, called Zeitgebers, most notably light signals received by the retina (Colwell et al., 1993). Ganglion cells in the retina send light information to the SCN through the retinohypothalamic tract (RHT). At the SCN, RHT terminals release glutamate, which binds to NMDA receptors on the postsynaptic SCN cells. This initiates a sharp increase in intracellular calcium, which triggers a series of intracellular changes, ultimately resetting the phase of the SCN clock. Studies have shown that brain-derived neurotrophic factor (BDNF), a growth factor, must concurrently activate its cognate TrkB receptors in order for NMDA receptor activation to produce SCN clock phase shifting (Prosser, 2001; Figure 1).

We hypothesize that a complex system of extracellular proteins, traditionally associated with the fibrinolytic

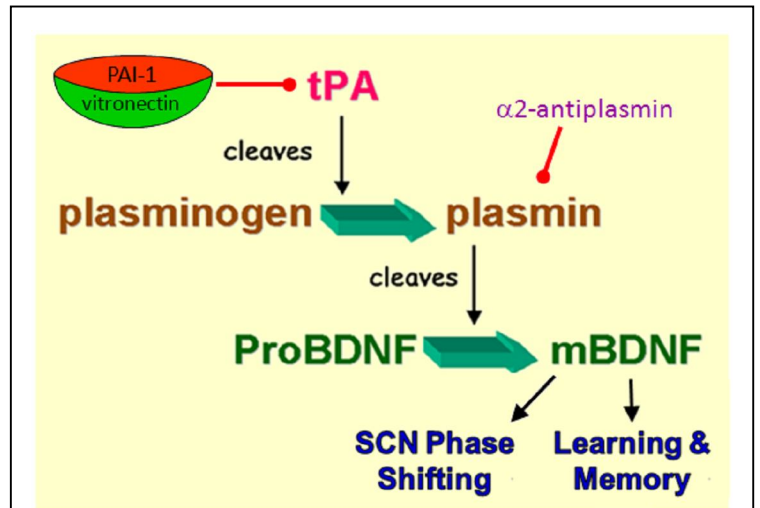
pathway and more recently shown to play a role in learning and memory in the hippocampus, also regulates how the clock responds to glutamatergic signaling in the SCN. Tissue plasminogen activator (tPA) cleaves plasminogen to form plasmin. Plasmin then cleaves proBDNF, the enzymatically inactive form of BDNF, to develop mBDNF, the enzymatically active form of BDNF (Figure 2). This pathway is involved in the processes that underlie learning and memory in the hippocampus (Pang et al., 2004). We are also interested in two inhibitors that modulate the fibrinolytic pathway in the plasma. First, plasminogen activator inhibitor 1 (PAI-1), stabilized by its cofactor vitronectin (VN; Podor et al., 2000), is the principle inhibitor of tPA in the



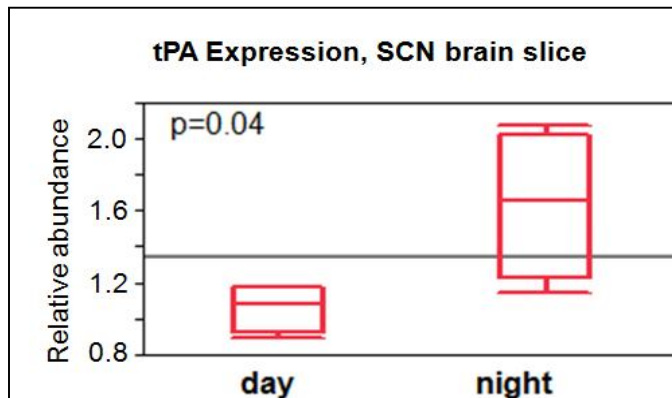
plasma (Neuhoff et al., 1999). Second,  $\alpha$ 2-antiplasmin inhibits plasmin activity (Collen and Wiman, 1978). The roles of these inhibitors have not been studied in the hippocampus.

Recent data in the Prosser lab indicate that tPA, PAI-1, plasmin, plasminogen, and VN are all expressed in the SCN. Further, these proteins and their inhibitors are involved in the modulation of photic phase shifts in SCN brain slices maintained *in vitro* (Mou et al., 2009). Furthermore, tPA, mBDNF, and PAI-1 protein levels all exhibit 24 hour rhythms in SCN brain slices. Most relevant to my research, his data show that tPA concentrations are significantly higher during the night (Figure 3),

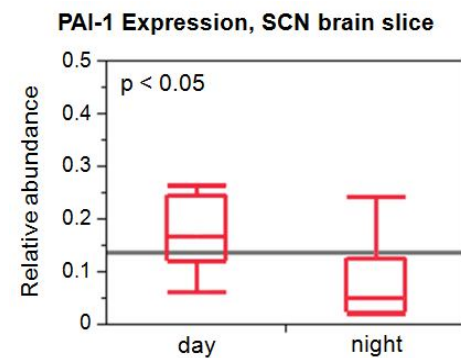
and that PAI-1 concentrations are significantly higher in the day (Figure 4; Mou et al, unpublished).



**Figure 2:** In the hippocampus, tissue plasminogen activator (tPA) activates BDNF by converting plasminogen to plasmin, which cleaves proBDNF to form mature BDNF (mBDNF). mBDNF enhances glutamate-induced late phase long term potentiation (L-LTP), which is involved in memory formation. tPA is inhibited by plasminogen activator inhibitor-1 (PAI-1), while vitronectin binds to and enhances PAI-1 activity. The mechanisms that underlie L-LTP and photic phase shifting are similar, and so we hypothesize that this pathway is involved in BDNF activation and, therefore, phase shifting in the SCN.



**Figure 3:** Data obtained from SCN brain slices. Designation of day and night were based on the light cycle in which the animal that provided the brain slice was housed. **Source: X. Mou**



**Figure 4:** Data obtained from SCN brain slices. Dark bars indicate night and are based on the light cycle in which the mouse was housed. **Source: X. Mou**

While these data on SCN clock phase resetting and protein expression rhythms give an indication of how these proteins function in the SCN, they do not provide the whole story. These proteins are secreted and

generally act extracellularly, sometimes bound to the extracellular matrix or cell surface proteins. The proteins they interact with largely determine their activity. Thus, protein expression in whole tissue samples only begins to describe their potential function in the SCN.

My project was to determine the temporal patterns of expression, and cellular localization, of tPA and PAI-1 in SCN2.2 cells working with post-doctoral fellow, Dr. Elizabeth Cantwell. The SCN2.2 cell line, an immortalized heterogeneous cell culture derived from fetal rat SCN (Earnest et al., 1999b), serves as an appropriate model for the rat SCN circadian clock due to several of its special characteristics. First, all proteins and mRNAs studied and published on in this cell line are expressed in the same manner as they are in the rat SCN (Earnest et al., 1999a, 1999b; Allen et al., 2001; Allen and Earnest, 2002; Hurst et al., 2002a, 2002b). Second, these cells can sustain endogenous circadian rhythms in culture (Earnest et al., 1999b) and can restore behavioral rhythmicity if they are implanted into an SCN-lesioned animal (Earnest et al., 1999a). Finally, SCN2.2 cells can synchronize rhythms of metabolic activity and gene expression in arrhythmic cells when kept in co-culture conditions (Allen et al., 2001). Using the SCN2.2 cell line allows us to refine our understanding of how these proteins function in the SCN by allowing us to differentiate their location between the culture media, which represents extracellular fluid, the cellular fraction, and the extracellular matrix (ECM). We hypothesize that at least some of the proteins are expressed at higher levels during the night than in the day, similar to the SCN brain slices also studied in our lab. However, there is little upon which to base speculation regarding the distribution of these proteins among the different compartments over the 24-hour cycle. Up until this point, most studies investigating photic phase shifting have focused on intercellular processes, so this is a novel line of research.

## **Methods**

### SCN2.2 Cell Cultures

My experiments utilize a heterogeneous immortalized cell line derived from the embryonic rat SCN called SCN2.2 cells. We obtained the cell line from Dr. David J. Earnest of Texas A&M University and developed our

own stock, which is frozen in cryotubes and kept in liquid nitrogen. We maintain the cultures according to his established protocols (Earnest et al., 1999a and personal communications).

To start a culture, cryotube stock is incubated in a water bath at 37°C for one to two minutes. Thawed stock is then pipetted into a fifteen milliliter Falcon tube already containing eight milliliters of warm growth media (Minimal Essential Media containing 20% fetal bovine serum (FBS), 0.4% glucose, 1% L-glutamine, 50 µg/mL gentamicin). The cryotube is rinsed with an additional one milliliter of media to remove any cells that remain, and this is transferred to the Falcon tube making the total volume ten milliliters. The Falcon tube is centrifuged (900 rpm, room temperature) for five minutes. The supernatant is then removed and the pellet is resuspended with a glass pipette in one milliliter of media. The resuspended cells are then transferred to a sixty millimeter Petri dish coated with laminin to encourage adherence. Three milliliters of warm medium is added to the dish, and it is incubated in a standard culture incubator (5% carbon dioxide, 37°C). The media is changed every twenty-four hours.

Under these conditions, the cells divide at a rapid rate. When the cells reach 100% confluence, meaning they cover the surface of the dish, we passage the cells. In the first passage, cells from the Petri dish are distributed, or seeded, into a flask that has roughly three times the area. In further passages, cells from one flask are seeded into three flasks. In this way, we are able to produce multiple flasks for experimentation from one cryotube of stock. To begin a passage, the confluent dish is rinsed with sterile Calcium-Magnesium Free buffer (CMF; phosphate buffered saline containing 0.2% glucose and 50 µg/mL gentamicin). Two milliliters of 0.05% Trypsin/EDTA is added to the dish, and it is incubated for one minute at 37°C, which causes the cells to lift from the surface of the dish. As they lift, the cells take on a spherical appearance. At this point, we add 0.2 milliliters of FBS and mix gently to stop the trypsinization. The cells are then triturated gently with a glass pipette until at least ninety-five percent of the cells are detached. The cells suspended in Trypsin/FBS are transferred to a fifteen milliliter Falcon tube and centrifuged (900 rpm; room temperature) for five minutes. The supernatant is discarded, and the cell pellet is resuspended in 1 mL of fresh growth media with a glass pipette. After adjusting the volume appropriately, 1 mL of resuspended cells in media is put into each flask, and

additional growth media is added to bring the flask volume to 10 mL. The dishes are incubated at 37°C, and the media is changed every 24 to 48 hours, depending on the rate of growth.

### Synchronization

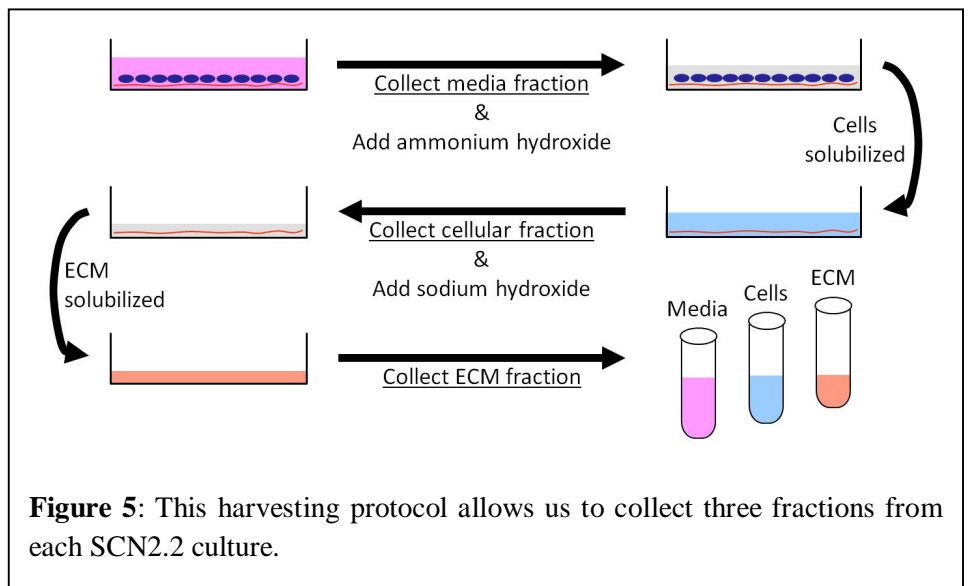
At the point where we have passaged nine dishes and they have reached about 75% confluence, we begin our synchronization protocol. Media is removed and replaced with growth media containing 5% FBS eight hours before synchronization. The dishes are then rinsed with CMF, and we add B-27 media (Neurobasal media containing 0.81% glucose, 1% L-glutamine, 50µg/mL gentamicin, and 2% B-27 nutritional supplement) containing 15 µM forskolin in 0.01% DMSO (old protocol) or 0.1% DMSO (new protocol). After incubating for two hours at 37°C, the flasks are rinsed with CMF and filled with 10 mL fresh B-27 media, except the flask to be harvested at the first timepoint, which is filled with 5 mL of fresh B-27 media.

### Harvesting

Three hours after synchronization ends, the timecourse of sample collection begins. Cultures are harvested at three-hour intervals over a twenty-four hour cycle, one flask at each timepoint. At the outset of each timepoint, the media in the flask to be harvested 3 hours later is replaced with five milliliters of fresh B-27.

Then, sample collection begins

(Figure 5). First, media is collected, cleared, and aliquotted into Eppendorf tubes. Second, the remaining culture is rinsed, and 0.02M ammonium hydroxide is applied to the remaining culture for ten minutes at room temperature to solubilize the cells remaining the



**Figure 5:** This harvesting protocol allows us to collect three fractions from each SCN2.2 culture.

flask. The cell fraction is collected, mixed, and aliquotted into Eppendorf tubes. Third, the remaining

extracellular matrix (ECM) is rinsed and solubilized in 1M sodium hydroxide. After manual agitation, pH is equilibrated with 1M acetic acid, and the ECM fraction is collected, mixed, and aliquotted into Eppendorf tubes. The three sample fractions are stored at -80°C until they are thawed for protein extraction.

### Protein Extraction

My experiments have utilized the cell fraction of the SCN2.2 cell line. Proteins are precipitated in 10% trichloroacetic acid (TCA). To accomplish this concentration, a ten percent volume of ice-cold 100% TCA in acetone is added to 50  $\mu$ L of each cell fraction collected over the timecourse. This solution is incubated for ten minutes on ice, and then centrifuged (17g, 4°C) for 30 minutes. The supernatant is discarded, and the protein pellet is rinsed with 250  $\mu$ L of ice- cold 1% TCA in acetone for 10 minutes on ice. The rinsed pellet is then centrifuged, as before, for 15 minutes. The 1% TCA rinse is repeated once. Next, the pellet is rinsed with 250  $\mu$ L of ice-cold acetone three times in the same manner as the 1% TCA rinses. Finally, the protein pellets are left overnight at room temperature to dry.

### Western Blotting Procedure

Protein in the dried pellets is reconstituted in loading buffer (80mM Tris, 2% SDS, 100mM Dithiothrietal, 10% glycerol, 0.01% bromophenol blue). A uniform volume of each sample is loaded into separate wells of an acrylamide gel (4% stacking, 10% resolving), as is a ladder, consisting of proteins of known molecular weight covalently bonded to visible dyes, and a purified protein control, appropriate to the protein I am going to visualize. Proteins in the samples are separated by molecular weight using gel electrophoresis. Because these proteins are negatively charged, and the current applied creates a negative pole at the top and a positive pole at the bottom, the proteins migrate through the pores of the acrylamide gel such that the larger proteins migrate slowly and stay close to the wells, and the smaller proteins move through the gel quickly and end up further from the wells. Proteins are transferred from the gel to a nitrocellulose membrane in an apparatus that also applies current, allowing them to migrate from the gel to the membrane, where they are crosslinked. Once proteins have been transferred, the membrane is subjected to standard Western blot techniques utilizing primary



antibodies generated against tPA and PAI-1. Secondary antibodies conjugated with horseradish peroxidase (HRP) allow me to visualize my protein by applying a chemiluminescent substrate, which, when cleaved by the HRP, emits visible light. By exposing photographic film to the membrane and then developing it, we get a permanent image of protein expression in the cell fraction of the SCN2.2 cell line. The same membranes are then probed using primary antibodies generated against actin, and these values are used to normalize protein data as discussed below.

### Data Analysis

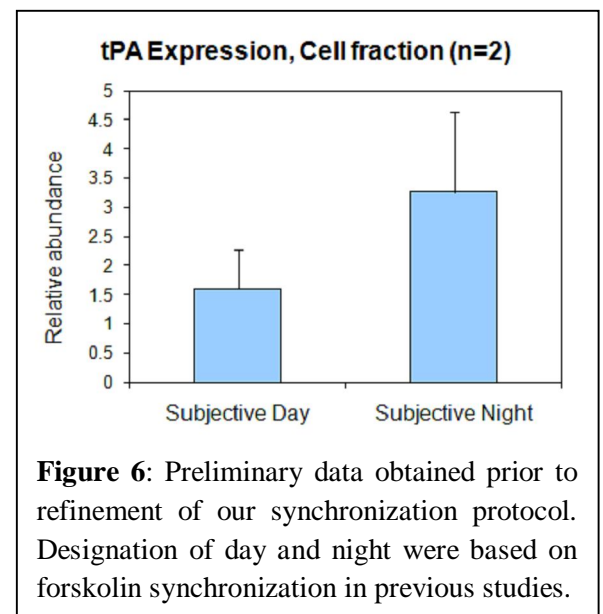
To analyze my data, I digitize films on a scanner and use the ImageJ program (NIH, Bethesda, MD) to quantify the density of bands representing protein expression. The data for each sample are normalized by expressing the values for the protein of interest and actin from each sample as a ratio. The data are stored on an Excel spreadsheet, and graphs are made from the data. Statistical analysis is performed on the JMP 8 statistical discovery software by SAS (Cary, NC). Significance is determined using a one-way analysis of variance (ANOVA), followed by Student's T post-hoc test.

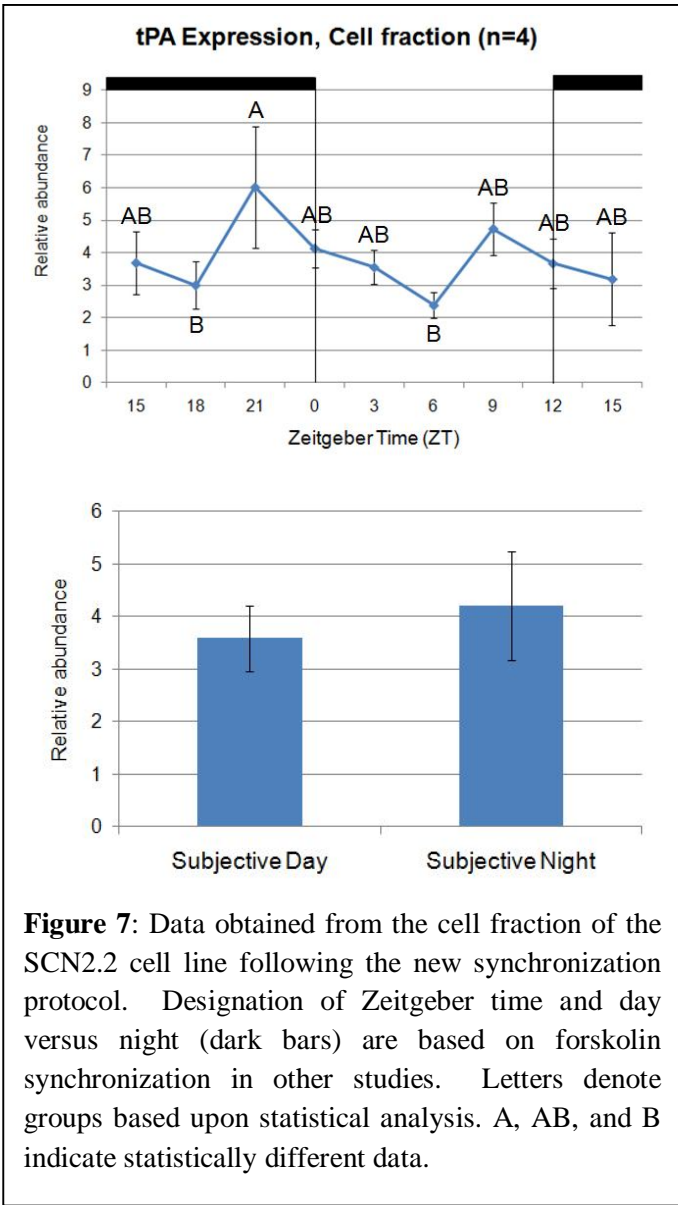
## **Results**

### tPA Expression in the SCN

Preliminary data were obtained from the cell fraction of the SCN2.2 cell line following the old synchronization protocol (Figure 6). Data from two Western blots support findings in the SCN brain slice; however, since statistical analysis is not appropriate in this case (with only 2 replicates), we cannot determine significance.

Data obtained under the new synchronization protocol indicates that there is a significant difference in tPA expression





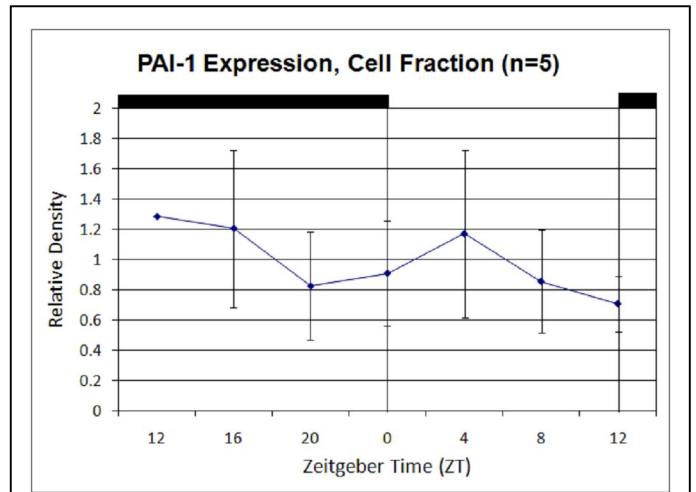
**Figure 7:** Data obtained from the cell fraction of the SCN2.2 cell line following the new synchronization protocol. Designation of Zeitgeber time and day versus night (dark bars) are based on forskolin synchronization in other studies. Letters denote groups based upon statistical analysis. A, AB, and B indicate statistically different data.

PAI-1 Expression in the SCN

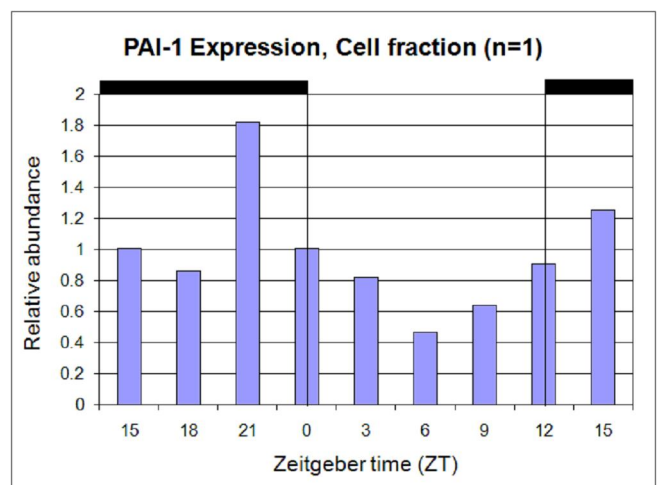
Preliminary data from the cell fraction of the SCN2.2 cell line obtained following the old synchronization protocol indicate that there is no significant difference in PAI-1 expression over the course of the 24-hour cycle (Figure 8).

Data obtained from the cell fraction following the new synchronization protocol are inconclusive at this

across time ( $p < 0.05$ ), in the cell fraction of the SCN2.2 cell line, Post-hoc analysis indicates that tPA expression at ZT21 (late night) is significantly higher than expression at ZT18 (mid-night;  $p = 0.016$ ) and ZT 6 (mid-day;  $p = 0.042$ ). However, we found no statistically significant difference in overall tPA expression from day to night (Figure 7).



**Figure 8:** Preliminary data obtained prior to refinement of our synchronization protocol. Black bars indicate night, designation based on previous studies.



**Figure 9:** Data obtained from the cell fraction of the SCN2.2 cell line following the new synchronization protocol. Black bars indicate night, designation based on previous studies.

time. This data show a high peak in PAI-1 expression during the late night (Figure 9). Because only one replication was successful, statistical analysis is not appropriate.

## Discussion

In this study, we have shown that, in the cell fraction of the SCN2.2 cell line, tPA protein expression varies significantly over the course of the day. PAI-1 expression, while not subjected to statistics, seems to peak in the late night. There are several aspects of these data that are quite important.

The data obtained from Xiang Mou indicates that tPA expression is significantly higher in the night (Figure 3), while PAI-1 levels are significantly higher during the day (Figure 4) in SCN brain slices. However, these data leave open questions as to where and when tPA and PAI-1 are expressed in the SCN. We chose to address this issue using the SCN2.2 culture system because we can research the localization of these proteins over the twenty-four hour cycle.

The SCN brain slice data (Figure 3) demonstrated that tPA is significantly higher in the night than in the day in SCN brain slices. However, according to our cell culture data (Figure 7), there is no statistically significant difference between tPA expression in the day versus the night. However, the data show a significant peak (Figure 7, peak A) toward the end of the night. When comparing these data with those from whole SCN tissue, this could mean that tPA is localized to the media or extracellular matrix strongly during the night.

While the SCN brain slice data and preliminary data reveal higher expression of tPA at night than in the day, our more recent cell culture data (Figure 7) did not support this pattern in the cell fraction. One reason for this discrepancy could be the procedural changes in the cell culture technique. We increased the volume of DMSO, a substance used to dissolve forskolin, which is hydrophobic. This was necessary because we discovered that the volume being used before was not dissolving all of the forskolin. If the forskolin does not dissolve completely, the cells will not synchronize. However, DMSO is toxic to the cells, so this could have affected the growth of the cells over the 24-hour time course, therefore affecting cells in the different flasks differently. While visual inspection of the cells indicated that they were healthy following the forskolin pulse,

there is a possibility that they were dying over time. Thus, we may have to reassess the synchronization process because as cells die, they may release tPA into the media, and this would skew the tPA expression data obtained from the media fraction. Therefore, tPA expression data from the media fraction should be highly informative as to this issue.

The fact that forskolin dissolves completely remains so important because we assume that forskolin synchronizes our cells as it did in other studies because the best indicator of what phase the cells are in is BDNF. In previous studies, BDNF expression was assessed by an ELISA kit; however, that kit was discontinued by the manufacturer. Adjusting our western blot procedure to quantify BDNF has been a challenge.

While PAI-1 expression in the SCN2.2 cell fractions has shown no rhythmicity, it might be rhythmic in one or both of the other two fractions. My data suggests that PAI-1 may be expressed in higher levels at night, but this does not fit with the SCN brain slice data. However, since PAI-1 is only active when bound to the extracellular matrix, we might find high PAI-1 levels a few hours later in the ECM and media where it is actually active. This would make sense, given that inhibition of tPA during the day would halt activation of BDNF, and it is during the day that the SCN cannot phase shift in response to light or glutamate.

Obviously, we need to continue our investigation of all three compartments with each protein to determine what is being expressed where. However, presence of these proteins is not the only issue: we also need to determine when tPA and PAI-1 are enzymatically active.

From a real-world standpoint, this research is extremely significant. If we understand how light phase shifts the clock, we may be able to find a way to make jet lag obsolete by modulating the proteins that play the most important roles in photic clock synchronization. Also, links have been found between BDNF expression and post-traumatic stress disorder (PTSD; Bremner 2006). If we can manipulate BDNF expression, that could help us develop a potential treatment for PTSD. Further, incorrect synchronization of the SCN has been linked to a wide variety of psychiatric disorders such as schizophrenia, depression, seasonal affective disorder. New treatments could be developed with a greater understanding of how the circadian system works.

## Conclusion

The preliminary data showed higher tPA expression in the SCN2.2 cells at night, but more recent data did not confirm this. Preliminary data also indicated no rhythm in PAI-1 expression in the SCN2.2 cells. The Western blot completed under the new synchronization protocol suggests higher levels at night, but it is based on only one replication. This research will be continued by further experimentation focusing on protein levels in the media and ECM fractions.

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